Characterization of an Immortalized Cell Line from a Patient with Epidermolytic Hyperkeratosis

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The most frequent mutation that causes the autosomal dominant skin disease epidermolytic hyperkeratosis (EHK) is an arginine to histidine substitution at position 10 in the 1A segment of the rod domain of keratin 10. As an initial step toward developing a strategy for treating EHK, a cell line, EH18–1, was established after keratinocytes derived from an EHK patient with this mutation were immortalized by a recombinant retrovirus encoding the E6 and E7 genes of human papillomavirus type 18. EH18–1 cells synthesize considerable amounts of keratin 10 mRNA and protein when maintained in either submerged cultures or in organotypic cultures. When grown in organotypic culture, EH18–1 cells form multiple layers and express keratin 10 and filaggrin predominantly in the upper layers. Thus, the EH18–1 cell line exhibits several morphological and biochemical markers of terminal epidermal differentiation. A semiquantitative reverse transcriptase polymerase chain reaction assay for keratin 10 mRNA was developed to distinguish between expression of the normal and the mutant alleles. The EH18–1 keratinocyte cell line will be useful in developing protocols for gene therapy of EHK that may be monitored by reverse transcriptase polymerase chain reaction of either allele. ***Key words: RT-PCR allele specific assay/immunohistochemistry/submerged and organotypic cultures/Arg to His mutation/keratin 10. J Invest Dermatol 106:385–390, 1996***

Epidermolytic hyperkeratosis (EHK) is an autosomal dominant human skin disease. In recent years, considerable progress has been made in elucidating the molecular basis of EHK (Steinert and Bale, 1993; Fuchs and Weber, 1994; Rothnagel and Roop, 1995), with mutations in the keratin 1 (K1) and keratin 10 (K10) genes identified as causing this disease (Rothnagel *et al*, 1992; Cheng *et al*, 1992; Chipev *et al*, 1992, 1994; Yang *et al*, 1994; McLean *et al*, 1994). Most of the cases of EHK identified can be attributed to single point nucleotide substitutions, which result in the incorporation of an inappropriate amino acid residue and of which an arginine to histidine substitution in position 10 of the 1A rod domain of the keratin 10 chain is most common.

EHK is characterized histologically by hyperproliferation, thickening of the cornified layers, and cytolysis in the suprabasal layers. Ultrastructurally, the suprabasal keratinocytes of affected patients show tonofilament aggregation and abnormal keratohyalin granules (Ishida-Yamamoto, 1992). Some of these features have been reproduced in the epidermis of transgenic mice carrying a truncated human keratin 10 (Fuchs *et al*, 1992)[1] or keratin 1 (Rothnagel *et al*, 1993) gene, but no EHK phenotype has been reported in transgenic animals for the types of single point mutations most commonly seen in human patients. It has proven more difficult, however, to study the disease *in vitro*. For example, when EHK keratinocytes from a patient were grown in monolayer cultures, the cells grew transiently but displayed either no changes in morphology (Ishida-Yamamoto, 1992) or only subtle differences in only about 5% of the cells (Huber *et al*, 1994).

In considering possible gene therapy strategies, it is important to note that the most common mutation of EHK (a G to A substitution leading to an Arg to His change) in the keratin 10 chain is not suitable for ribozyme technology because the requirement for the ribozyme consensus sequence (. . .NNTHNN. . . , H = A,C,T) (Sullivan, 1994) is not met by the particular sequence at the mutated site (. . .ACC*A*CC. . .). Of the other approaches such as gene transfer into keratinocytes (Fenjves, 1994), the recombinant virus delivery of normal K10 cDNA may not be successful since the mutant K10 allele will be still expressed and act in a dominant negative mode. On the other hand, anti-sense technologies targeting predominantly the mutant transcripts may have therapeutic value.

We have reasoned that the development of a cell line on which different therapeutic protocols can be tested would be very useful. In this paper, we describe an immortalized keratinocyte cell line derived from an EHK patient by use of a recombinant retrovirus encoding the E6 and E7 genes of human papillomavirus type 18. We have characterized the biological properties of the established cell line (EH18-1) and present a reverse transcriptase polymerase chain reaction (RT-PCR) assay that can distinguish between normal and mutant K10 mRNA expression. EH18-1 cells express the normal and mutant K10 mRNAs at levels comparable to normal keratinocytes grown in the same conditions. Thus, this cell line and the use of the specific RT-PCR assay will now allow detailed studies on possible treatment regimens.

MATERIALS AND METHODS

Patients The individuals are from a family in which two persons have EHK, a mother who represents a new sporadic mutation for the disease, and her affected child. This case has been classified as type NPS-3, distinguished

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[1] Porter RM, Leitgeb S, Melton DW, Swensson O, Eady RAJ, Magin TM: *J Invest Dermatol* 104:555, 1995 (abstr.)

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by erythroderma and fine white scaling (DiGiovanna and Bale, 1994). The mutation identified in the patient's DNA was a G to A transition in one of the alleles, leading to an arginine to histidine substitution in position 10 of the 1A rod domain of keratin 10. A 3-mm punch biopsy was taken from an involved region of the skin of the mother. DNA for PCR analyses was also recovered from the affected mother and daughter, and the unaffected father.

Keratinocyte Cultures Submerged cultures were grown in the serum-free keratinocyte medium KGM (Clonetics, San Diego, CA), supplemented with 60 μg/ml bovine pituitary extract at 0.05 mM $CaCl_2$ (referred to as low calcium), or at 1.2 mM (high calcium). The high calcium was introduced after the cells reached confluence. Cells were harvested 1–5 d after reaching confluence. Cryopreserved normal human epidermal keratinocytes were obtained from Clonetics. Keratinocytes were also grown from adult skin biopsy from a normal adult.

Primary Cell Culture and Retrovirus Infection The sample of skin from the mother was placed in Dulbecco's modified Eagle's medium containing 0.25% collagenase (Boehringer Mannheim) and incubated at 37°C for 16 h. Tissue was dispersed by gentle pipetting, and several primary cultures were established in KGM. When cultures were 40% confluent, cells were infected with a replication defective retrovirus (Woodworth *et al*, 1992) encoding either the HPV-18 E6 and E7 genes plus the neomycin resistance gene, or a virus encoding only the neomycin gene (negative control). Infected cultures were treated with geneticin (400 μg/ml for 48 h) to eliminate uninfected cells. Individual colonies were pooled and subcultured until the negative control cells became senescent (approximately 30 population doublings).

Organotypic Cultures Collagen rafts embedded with NIH3T3 cells were prepared as described (McCance *et al*, 1988). Approximately 10^6 keratinocytes were layered on the raft and maintained at the air-liquid interface for 10 d before being embedded in OCT compound (Miles Laboratories, Elkhart, IN) and snap frozen for histology.

Total RNA Total RNA was isolated from rapidly frozen foreskin epidermis, from submerged cell cultures, or from raft cultures using the Trizol reagent (Gibco-BRL, Bethesda, MD).

Amplification of RNA and DNA Thermostable rTth DNA polymerase (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ) was used to reverse transcribe 50–100 ng total RNA/10 μl reaction volume. Amplification was performed in the same tube according to the manufacturer's conditions with 2 mM $MgCl_2$ usually for up to 30 cycles with denaturing at 95°C for 1 min, annealing at 60°C and extension for 90 sec at 72°C. Plasmid cDNA clones (0.1–1 ng) were amplified in separate tubes. Genomic DNA (300–500 ng/50 μl) was amplified with Taq polymerase (Perkin Elmer) at the same temperatures as above but for 35 cycles. To monitor the amplification α-[^{32}P-dCTP] (2–5 μCi, 3000 Ci/mmol; New England Nuclear, Boston, MA) was included in the reaction mixtures containing one-eighth of the standard amount of unlabeled dCTP. The PCR products were analyzed on precast 6% TBE-urea polyacrylamide gels (Novex, San Diego, CA). The dried gels were scanned on a PhosporImager and analyzed with the ImageQuant software package (Molecular Dynamics, Sunnyvale, CA).

Primers Used in RT-PCR and PCR

Keratin 10 gene: The primers used in RT-PCR and PCR for the K10 gene were as follows: **R**$^-$ (beginning of exon 2, 3144–3173 bp according to Rieger and Franke [1988]): 5′-ATGTTGGCATTATCAGTTGTTAGGTTGAGA-3′; **H**$^-$ (end of exon 1, 2261–2290 bp): 5′-GGTCATCGATGGTTTTGTAGTATTTGCTG-3′; **MSC**$^+$ (2109–2139 bp): 5′-GAAAAAGTAACCATGCAGAATCTGAATGGCC-3′ (the normal K10 sequence has an A in place of the G).

Keratin 1 gene: Primers for the K1 gene were **K1**$^+$ (beginning of exon 2, codons for amino acids 199–207 according to Johnson *et al* [1985]): 5′-TCCTGGAGCAGCAGAACCAGGTAC-3′; **K1**$^-$ (exon 3, codons 279–287): 5′-CTTGATGGTCACAAATTCATTCTCT-3′.

Glyceraldehyde phosphate dehydrogenase gene: Primers for the glyceraldehyde phosphate dehydrogenase (GAPDH) gene (from GenBank accession number M33197): **GAPDH**$^+$ (bp 71–94): 5′-GAAGGTCGGAGTCAACGGATTTG-3′; **GAPDH**$^-$ (bp 234–257): 5′-GCCATGGAATTTGCCATGGGTGGA-3′.

Cloning of the Normal and Mutant Allele PCR Products RT-PCR products from EH18–1 cell RNA obtained with keratin 10 primers **R**$^-$ and **MSC**$^+$ were extracted from a 2% agarose gel with GeneClean (Bio101 Inc., LaJolla, CA) and cloned directly in the 3.9-kb TA cloning vector pCR II (Invitrogen Corporation, San Diego, CA). Selection was done by lacZ complementation and Southern blot hybridization.

Introduction of an *Msc* I Site to Distinguish Between the Mutant and Normal K10 Alleles The sequence of the normal allele around the mutation site is TGA*CCGCC* (an *Aci* I site is in italics). The mutant allele has the corresponding sequence TGA*CCACC* (the mutated nucleotide is in italics; the *Aci* I site is destroyed). The primer **Msc**$^+$ introduces a G (instead of the A) in the PCR products from both alleles. The normal allele PCR product now has the sequence TG*gCCGCC* (the mismatch is shown in a lower case letter; the *Aci* I site is retained; no *Msc* I site is present). The corresponding mutant allele PCR product has the sequence *TGgCCACC* (an *Msc* I site [in italics] is created by the mismatched g and the mutation *A*; the *Aci* I site is not present).

Restriction Enzyme Digestion of PCR Products Digestion with restriction endonucleases *Aci* I and *Msc* I (New England Biolabs, Beverly, MA) was performed with aliquots of the PCR reaction without purification and by adjusting the $MgCl_2$, Tris, and NaCl concentrations for the corresponding enzymes.

Immunohistochemistry Keratinocytes grown as submerged cultures on coverslips were fixed for 5 min in methanol:acetone (1:1) solution. Cells were rehydrated, blocked with nonimmune serum, and rinsed. Frozen sections of the airlifted cultures were treated with ice-cold acetone, rinsed, and blocked with 2% gelatin. Incubation with monospecific antibodies was for 1 h. To visualize the primary antibodies, goat $F(ab')_2$ anti-mouse or anti-rabbit IgG fluorescein isothiocyanate conjugates (Tago Incorporated, Burlingame, CA) and goat anti-rabbit IgG rhodamine conjugates (Cappel, Durham, NC) were used. Rinsing at this step was with phosphate-buffered saline containing 0.5% Tween-20. Indirect immunofluorescence microscopy was done on a Olympus System Microscope Model BH-2 and Olympus photomicrographic system model PM-10ADS.

Antibodies They were used in 1:100 dilution and were specific to: anti-human keratin 10 (mouse, monoclonal, DE-K10), and anti-human keratins 5+8 (mouse, monoclonal RCK102; Cappel, Durham, NC); anti-human filaggrin (mouse, monoclonal; Biomedical Technologies Inc., Stoughton, MA); anti-mouse keratin 5 (rabbit, polyclonal, affinity purified, a kind gift from S. Yuspa, NCI, NIH, Bethesda, MD); and anti-human keratin 10 (rabbit, polyclonal, elicited against the carboxyl-terminal peptide [Roop *et al*, 1984]).

RESULTS AND DISCUSSION

Establishment of the EH18–1 Cell Line A 3-mm skin biopsy was used to initiate primary cultures of epidermal keratinocytes from a patient with EHK, carrying an arginine to histidine substitution in position 10 of the 1A rod domain segment of one allele of keratin 10. After infection with retroviruses encoding HPV-18 E6 and E7 proteins and selection by geneticin to eliminate uninfected cells, approximately 10 colonies emerged. Initially, these cells grew poorly, and attempts to isolate a clonal cell line failed. Therefore, individual colonies were pooled and maintained over a period of 3 mo, during which time the cells entered a period of crisis. After 10–12 wk, colonies of rapidly growing cells appeared. These cultures were subpassaged and frozen for future use. Cells from passages 14 to 26 were used in the analyses described here.

Synthesis of K1 and K10 mRNAs in the Immortalized Cell Line EH18–1 K1 and K10 are early markers of terminal differentiation in epidermal keratinocytes. Therefore, we examined the levels of K1 and K10 mRNA expression in the cell line grown as either submerged or organotypic cultures. The results of reverse transcription and amplification with keratin specific primers and equal amounts of total RNA are shown in **Fig 1**. In low-calcium conditions, the levels of K10 and K1 mRNAs in EH18–1 cells growing in submerged culture (**Fig 1**, *lane 1*), or organotypic culture (**Fig 1**, *lanes 2 and 3*, for passages 23 and 26, respectively) were comparable to those of cultures of normal keratinocytes (**Fig 1**, *lane 4*) and foreskin epidermis (**Fig 1**, *lane 5*). Thus, the immortalized cells express a differentiated phenotype while they continue to proliferate. The pattern of K10 and K1 mRNA expression did not change over the 13 successive passages of EH18–1 explored. The E6 and E7 genes have been reported to stimulate proliferation but delay and alter the differentiation program in epithelial cells by diminishing the expression of such differentiation markers as K1 (Woodworth *et al*, 1992; McCance *et al*, 1988). The EH18–1 cells, however, maintain a differentiation

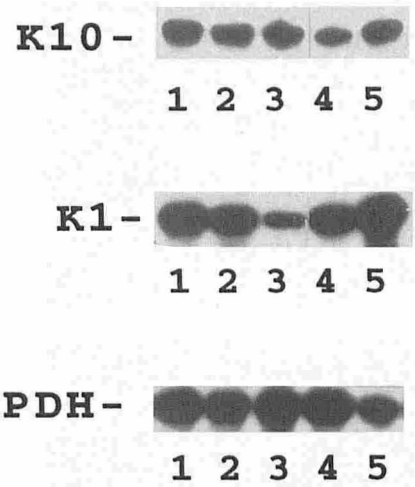

Figure 1. Expression of keratin 1 and 10 mRNAs in EH18–1 cells and normal epidermal keratinocytes. Equal amounts (100 ng) of total RNA were reverse transcribed and amplified in 50 μl. One-tenth of the products were resolved on Novex 6% TBE-urea polyacrylamide gels. *Lane 1*, EH18–1 keratinocytes in submerged cultures (passage 18); *lanes 2 and 3*, organotypic cultures of EH18–1 cells (passages 23 and 26, respectively); *lane 4*, normal human foreskin epidermal keratinocytes; *lane 5*, normal human foreskin epidermis. All primers amplified across introns and no separate DNase treatment was needed. The minus primers were used both for reverse transcription and amplification. The keratin 10 primers $\mathbf{R}^-$ and $\mathbf{MSC}^+$ give a 224-bp product; the keratin 1 primers $\mathbf{K1}^-$ and $\mathbf{K1}^+$ gave a 270 bp product; the GAPDH products are 163-bp long and are used as an RNA concentration reference. These three reactions were performed separately on the same batches of RNA.

program as shown by the continued high expression of K10 and K1 mRNA, and therefore may be very useful for studying the regulation of K10 and K1 mRNA and protein expression.

The EH18–1 Cells Synthesize K10 Protein After submerged cultures of EH18–1 cells achieved confluency in low-calcium conditions, some cells began to stratify as has been reported for normal epidermal keratinocytes (Poumay and Pittelkow, 1995). In high calcium conditions, the EH18–1 cells also behaved similarly to normal keratinocytes in the sense that the stratified cells were easily identifiable by their large polygonal shapes.

In cultures of normal human keratinocytes grown in low calcium medium, both the monolayer and stratified cells expressed K5 **(Fig 2*A*)**. About 3% of the total cells became stratified (as seen in Huber *et al*, 1995), almost all of which expressed K10 **(Fig 2*B,C*)** or filaggrin **(Fig 2*D*)**. Several days at confluence seemed to promote K10 expression in low calcium, confirming the results of Poumay and Pittelkow (1995). Likewise, all EH18–1 cells expressed K5 **(Fig 2*E*)**. In this case, more than 20% of the cells were stratified, essentially all of which expressed K10 **(Fig 2*F,G*)**, and some expressed filaggrin **(Fig 2*H*)**. Similar results were obtained when the cultures were grown in high-calcium medium after achieving confluency (data not shown). We found that after 4 d in high calcium more than 50% of the EH18–1 cells became stratified, of which more than 90% expressed K10. In contrast, only about 30% of normal keratinocytes became stratified in this time and expressed K10, in confirmation of previous work (Huber *et al*, 1994). It is presently unclear whether the higher levels of K10 in the EH18–1 cell line are because the K10 mutant allele promotes a hyperproliferation-like response as seen in EHK *in vivo*, or is due to an undefined alteration associated with immortalization. In this regard, an HPV-18 immortalized cell line, KC18-RV1, derived from normal foreskin keratinocytes described previously (Woodworth *et al*, 1992), also expressed K10 but also to a smaller extent than the EH18–1 cells (data not shown). Western blotting analyses (data not shown) confirmed the higher level of expression of K10 in EH18–1 cells in comparison to normal keratinocytes and KC18-RV1 cells grown under the same conditions.

In organotypic cultures, the EH18–1 cells stratified forming multiple layers (hematoxylin and eosin staining data, not shown; **Fig 2*I,J*)** which did not show differences in morphology in contrast to normal organotypic keratinocyte cultures. In this way, the EH18–1 organotypic culture more closely resembled the organotypic cultures of KC18-RV1—normal human keratinocytes immortalized with HPV18 (Woodworth *et al*, 1992). The suprabasal layers of organotypic cultures of EH18–1 cells, however, were more strongly positive for K10 **(Fig 2*I*)** or filaggrin **(Fig 2*J*)** expression than the lowermost cells, and in this way resemble normal organotypic cultures.

Taken together, these data indicate that the EH18–1 cells stratify and express the differentiation markers K1 and K10 while they continue to proliferate. This makes them ideally suitable for *in vitro* studies.

Cultured EHK patient keratinocytes were reported to show doughnut-shape–like keratin 10 positive staining in a few (about 5%) of the cells, perhaps indicative of an abnormal keratin intermediate filament cytoskeleton due to the mutant allele (Huber *et al*, 1994). We have observed similar staining in EH18–1 cells, and in the KC18-RV1 cell line (not shown), which was derived from normal foreskin keratinocytes (Woodworth *et al*, 1992). Thus, this rare ultrastructural feature may not be a unique characteristic of EHK keratinocytes.

Accordingly, instead of looking for an ultrastructural feature that might distinguish a cell line carrying a mutant keratin allele, we have developed a more quantitative RT-PCR assay specifically to distinguish between and measure the expression of the mutant and normal alleles.

An Assay to Distinguish between Normal and Mutant Allele Expression of K10 mRNA The patient chosen for use in this study carried the most common mutation so far observed in EHK, a G→A change leading to an arginine to histidine substitution in keratin 10. This mutation destroys an *Aci* I restriction enzyme site in the normal allele, so that the mutant allele can be identified [7,8,10]. Due to the technical problem of incomplete digestion, however, the loss of a restriction site does not always provide an unambiguous assay. To assess directly the expression of the mutant allele, we designed a primer ($\mathbf{Msc}^+$, see *Materials and Methods*) that creates an *Msc* I site (TGG/CCA) in the amplified product of the mutant allele, while the product of the normal allele retains only the *Aci* I site (C/CGC). By digesting separately with these two enzymes, complementary and direct information is obtained about the abundance of mRNA from each allele. We estimated the amount of the digested products from one allele and compared them with the undigested products from the other by densitometry of the gels.

Total RNA was isolated from secondary cultures of the patient cells that were derived from the original biopsy, and was analyzed in parallel with RNA from EH18–1 cells from either submerged or organotypic cultures. When RT-PCR with the keratin 10 primers $\mathbf{R}^-$ and $\mathbf{Msc}^+$, and restriction enzyme digestion was performed, we observed expression of both K10 alleles **(Fig 3*A*)**. *Aci* I digested the amplification product of the normal allele (224 bp) to a shorter length (194 bp), while the mutant product remained intact (224 bp). Conversely, *Msc* I cleaved only the PCR product of the mutant allele (to 195 bp). Plasmid clones of the normal and mutant allele cDNA were amplified in the same series either alone or mixed in different ratios **(Fig 3*B*)**. The PCR conditions were optimized for reproducible and quantitative results. We found that excess RNA or plasmid DNA led to saturation and even inhibition of the amplification. *Aci* I digestion was not always complete and yielded inconsistent results, while *Msc* I digestion was more reliable.

An unexpected result was the reproducible predominance of K10 mRNA expression from the mutant allele in the EH18–1 cells **(Fig**

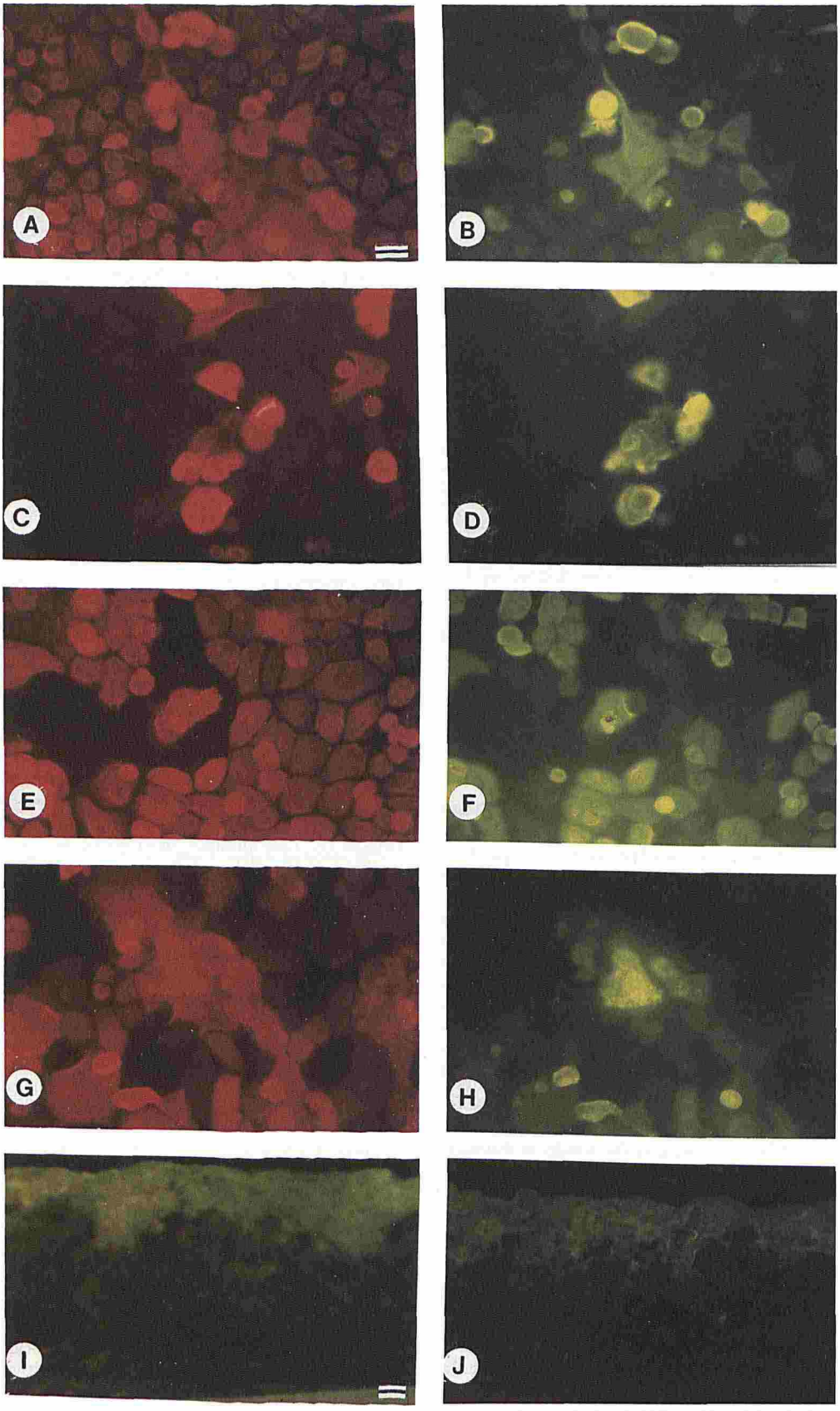

Figure 2. Immunohistochemical analyses of EH18–1 cells and normal epidermal keratinocytes. *A–D*) Submerged cultures of normal foreskin epidermal keratinocytes. *E–H*) Submerged cultures of EH18–1 keratinocytes. *I,J*) Organotypic cultures of EH18–1 cells. The pairs *A* and *B*, C and *D*, *E* and *F*, and G and *H* are from the same fields. Cells grown on slides or 5-μm frozen sections of organotypic rafts were incubated with: *A,E*) A rabbit anti-mouse keratin 5 antibody which also reacts against human keratin 5; *C,G,I*) A rabbit anti-human keratin 10 antibody; *B,F*) A mouse anti-human keratin 10 antibody; *D,H,J*) A mouse anti-human filaggrin antibody. The fields were visualized using fluorescein isothiocyanate–conjugated anti-rabbit or anti-mouse secondary antibodies and an anti-rabbit rhodamine-conjugated antibody. *Scale bars*: *A–H*) 15 μm; *I,J*) 30 μm.

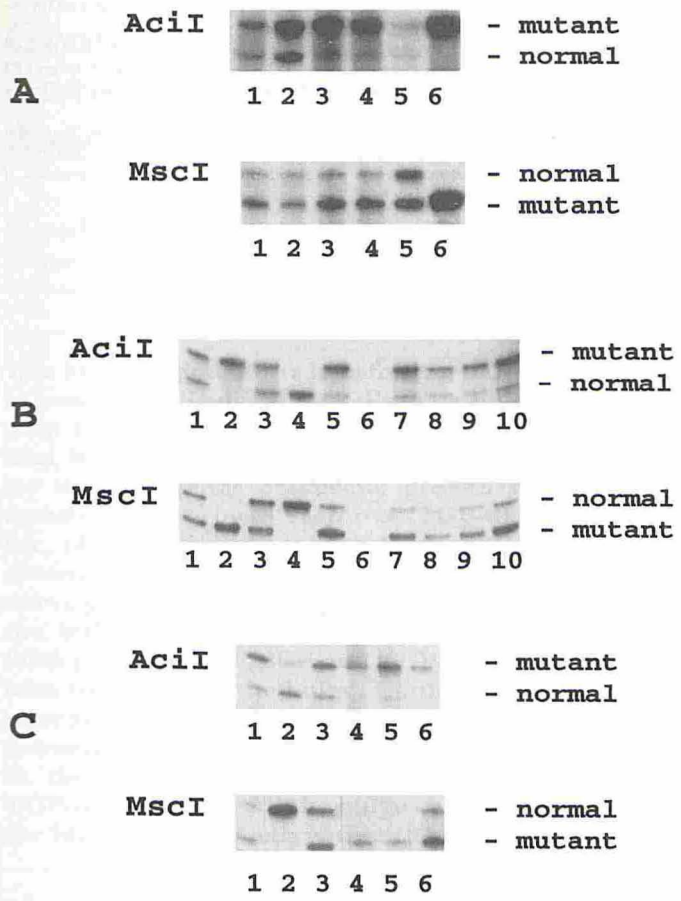

Figure 3. Keratin 10 RNA expression of the mutant and normal alleles. RT-PCR was performed for 30 cycles with incorporation of α-^{32}P-dCTP. One-tenth of the products were digested with restriction endonucleases *Aci* I (which reduces the length of the product of the normal allele from 224 to 194 bp), and *Msc* I (which reduces the length of the product from 224 to 195 bp). The digestion products were analyzed on Novex 6% TBE-urea polyacrylamide gels. *A*) *Lanes 1* and *2*, cultured cells from the patient (passages 4 and 5, respectively); *lane 3*, EH18–1 cells grown in submerged conditions; *lanes 4* and *5*, organotypic cultures of passages 23 and 26, respectively, of EH18–1 cells; *lane 6*, 1 ng of a mutant cDNA clone amplified in the same series. *B*) *Lanes 1* through *5*, normal (N) and mutant (M) cDNA clones in different mixtures: *lane 1*, N and M, 1 ng each; *lane 2*, M only, 1 ng; *lane 3*, N and M, 0.5 ng each; *lane 4*, N only, 0.5 ng; *lane 5*, N and M, 0.5 and 1.5 ng, respectively. These mixtures were amplified in parallel with EH18–1 cell RNA from: submerged cultures (*lanes 8* and *9*, passages 18 and 24, respectively); or organotypic cultures (*lanes 7* and *10*, passages 23 and 26, respectively). *Lane 6*, control in which no RNA or DNA was added in the reaction mixture. *C*) Presence of mutant and normal alleles in the genomic DNA of family members and EH18–1 cells. Amplification was for 35 cycles with primers **Msc**$^+$ and **H**$^-$. The PCR products are from the following DNA sources: *lane 1*, affected mother; *lane 2*, unaffected father; *lane 3*, affected daughter; *lanes 4* and *5*, EH18–1 cells (passages 18 and 24, respectively); *lane 6*, a mixture of normal and mutant cDNA clones (1 and 2 ng, respectively) amplified in parallel.

3A). Specifically, the EH18–1 cells contained three times more mRNA from the mutant allele than the normal allele. Similarly, a ratio of about 1.5:1 was observed in RNA from keratinocytes excised from the patient (**Fig 3*A***, *lanes 1,2*). One possibility is that this bias is an artifact of the RT-PCR assay. To exclude this, we used various stoichiometric mixtures of the normal and mutant plasmid clones. Densitometric analyses showed that the densities of the bands reproduced the initial mixing ratio of the templates (**Fig 3*B***) with a standard error of 30%. Thus, amplification of the mRNA from the normal and mutant alleles for up to but not more than 30 cycles did not alter the pre-existing ratio of these two alleles. Similar results were obtained with premixed genomic DNA from the two parents with different allele ratios. In this case, the assay was again sensitive to the allele presentation (not shown). We tested whether there is more DNA for the mutant K10 allele in the cell line. Quantitation of the PCR products using as template genomic DNA of the EH18–1 cells (**Fig 3*C***, *lanes 4,5*) showed an almost 2:1 predominance of the mutant allele relative to the normal allele. This bias was already present to about a 30% excess in the patient cells before transformation (**Fig 3*C***, *lanes 1,3* for affected mother and daughter). Thus, these DNA data parallel the RNA data. The reason for the elevated DNA levels is, however, not yet clear, but the enhancement following transformation may be due to chromosomal rearrangements, and because the EH18–1 cell line is not a single clone.

In conclusion, here we describe an immortalized cell line with a mutation in one of the keratin 10 alleles. The analyses performed reveal that the cells produce keratin 10 mRNA and protein in both submerged and organotypic culture systems. The keratin 10 protein expression was suprabasal and similar to that in normal keratinocytes in culture. A semiquantitative RT-PCR assay was developed that can be used to monitor the relative mutant and normal mRNA levels in response to possible different therapeutic gene therapy approaches.

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